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To identify genes misregulated in the final stages of breast carcinogenesis, we performed differential display to compare the gene expression patterns of the human tumorigenic mammary epithelial cells, HMT-3522-T4-2, with that of their immediate pre-malignant progenitors, HMT-3522-S2. We identified a novel gene, called AZU-1, that was abundantly expressed in non- and pre-malignant cells and tissues but was appreciably reduced in breast tumor cell types and in primary tumors. The AZU-1 gene encodes an acidic 571 amino acid protein containing at least two structurally distinct domains with potential protein-binding functions: an N-terminal serine and proline-rich domain with a predicted Ig-like fold and a C-terminal coiled-coil domain. Re-expression of AZU-1 in T4-2 cells was sufficient to reduce their malignant phenotype substantially, both in culture and *in vivo*. Reversion of the tumorigenic phenotype of T4-2 cells, by other means described previously, also was accompanied by the re-expression of AZU-1. These results indicate that AZU-1 is a candidate breast tumor suppressor that may exert its effects by promoting correct tissue morphogenesis.

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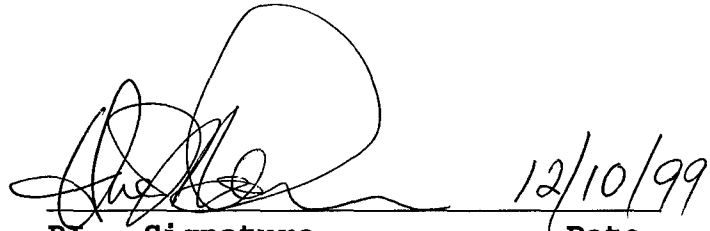

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ABSTRACT

Message levels of premalignant (S2) and tumorigenic (T4-2) cells, two sublines of a human breast cancer series, were compared to identify molecules marking the final events in malignant transformation. A novel gene AZ-1 expressed at a >10-fold higher level in S2 than in T4-2 cells was isolated. While AZ-1 message was present in nonmalignant breast cell lines, primary luminal epithelial cells, and cells from reduction mammoplasty, it was drastically downregulated or absent in ten mammary carcinoma cell lines and four breast carcinoma biopsies. Ectopically-expressed AZ-1 in T4-2 cells reverted them to a normal-like phenotype in a three-dimensional basement membrane culture. Interestingly, upregulation of AZ-1 message was also correlated with phenotypic normalization of breast tumor cells in a β1-integrin reversion system. Furthermore, AZ-1 stably- transfected T4-2 cells were 80% less invasive and clonogenic in matrigel invasion and colony-forming assays, respectively, than the vector transfectants. Second structure predictions indicated that C-terminus of AZ-1 is homologous to the rod domain of plakin family, the versatile organizers of cytoskeletal architecture. These data suggest that AZ-1 may participate in the cytoskeletal organization of breast epithelial cells and AZ-1 may provide a link to the understanding of the abnormal cell-cell and cell-extracellular matrix interactions in the breast tumor cells.

INTRODUCTION

Human breast cancer is thought to derive from the stepwise transformation of the luminal epithelial cells of the ducts and terminal lobular units (1,2). This has led to the conclusion that mutational events are critical to the genesis of the transformed phenotype. Mutations studied include the amplification or aberrant expression of the proto-oncogenes: c-*myc*, c-*erb*B2, *int*-2/*hst*-1 and infrequently H-*ras*, as well as, inactivation or deletion of tumor suppressor genes such as p*53*, *Rb*-1, and BRCA1 (3). Despite these clinical correlations, at present it is not yet possible to ascribe a pivotal role for a specific genetic mutation in breast cancer aetiology. To clarify this issue, it would be desirable to follow the progressive changes until malignancy ensue in the tissue *in vivo*. However, the time-course of breast tumor evolution can be quite long, taking anywhere from 5-30 years to develop. To follow the development of carcinoma in situ to invasive carcinoma, epidemiological protocols must span periods of 10-15 years (4). This precludes the feasibility of conducting meaningful and reproducible human studies and emphasizes the need to develop appropriate experimental models of human breast cancer progression in culture.

It is now widely accepted that extracellular matrix (ECM) is a key component of tissue microenvironment playing a determinant role in functional differentiation of developing and adult epithelia (5-7). In the mammary gland, extensive data exist showing that basement membrane components regulate the morphological and functional differentiation of mammary epithelial cells in culture and in vivo (8-10). In addition, altered interactions with ECM have been observed in mammary tumor development, emphasizing the importance of

microenvironmental regulation in normal development and malignancy (11-13). Signals provided to mammary epithelium by basement membrane may be mediated by integrins, the transmembrane heterodimeric cell-surface receptors that link ECM to structural and functional elements within the cell (14-16). Several integrin receptors for laminin, the main component of basement membrane including $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 6\beta 1$ and $\alpha 6\beta 4$ are expressed in normal human mammary epithelium. Altered expression of these receptors is a common occurrence in breast tumors (17-19). The disrupted tissue architecture observed in mammary adenocarcinoma is also frequently associated with alterations in integrin heterodimer profiles (20,21). Changes in $\beta 1$-, $\beta 4$-, $\alpha 2$-, $\alpha 3$- and $\alpha 6$-intergrins have been reported for mammary tumor cell lines and in tissue sections, and were shown to be associated with tissue disorganization, loss of polarity, increased tumor aggressiveness, and metastasis (22). Integrins are known to possess intrinsic kinase activity and to require associated molecules for signaling. The emerging concept is that integrin cooperatively works with linked kinase, or growth factor receptors, or also through their interactions with the cytoskeletal components. However, a relationship between altered signal transduction via integrins and the adherens junction pathways, and its relevance to the origin of the tumor phenotype has not been directly examined. This is mainly due to the lack of appropriate model systems in which such changes can be studied (23).

We have thus taken advantage of a unique epithelial cell model of breast cancer developed by Briand and coworkers (24,25). The HMT-3522 breast cancer series was established under chemically defined conditions from a breast biopsy of a woman with a nonmalignant breast lesion (26). The established cell line S-1 is entirely dependent on exogenous epidermal growth factor (EGF). In passage 118, cells were adapted to grow in medium without EGF and a new growth-transformed subline S-2 cells was generated and propagated at a high growth rate without exogenous EGF (27). A highly dramatic shift in phenotype was observed in passage 238 when the S-2 cells became tumorigenic in nude mice. After two mouse-culture passages, the resulting malignant transformed cell line (T4-2) was refractory to the growth-modulating effect of EGF and presented an extra copy of a chromosome marker, 7q- (25). These three cell lines (S-1, nonmalignant, S-2, premalignant, and T4-2, tumor) one originating from the other by spontaneous genetic events, therefore, provide a unique tool for addressing the carcinogenic event and particularly for us the altered ECM-signaling pathways through integrins involved in malignant conversion in the breast.

In addition to elucidating integrin signaling, we have also exploited molecular approach to search for candidate genes that might be involved in cellular transformation. Differential display originally described by Liang and Pardee (28) offers a powerful tool for this endeavor. The method is based upon comparison of mRNAs expressed in two or more cell populations by running their reverse transcribed and radioactively-labeled PCR products on sequencing gels in adjacent lanes. The bands revealing detectable differences between cell populations are cut and the cDNAs are eluted. After PCR re-amplification, the eluted cDNAs can be directly used a s probes in northern blots for verification and in subsequent recovery of the full-length clones from cDNA libraries. By using suitably chosen PCR primers, the majority of the cDNAs can be displayed as 100-600 bp fragments. Several candidate tumor suppressor genes in breast cancer

including α6 integrin were identified by this method (29).

Using this differential display approach, we have isolated a novel tumor suppressor candidate gene AZ-1. Sequence analyses and secondary structure predictions indicated that C-terminal coiled-coil domain of AZ-1 protein showed sequence homology to the rod domain of plakin family proteins. Plakin family comprises four sequence-related proteins: desmoplakin, plectin, bullous pemphigoid antigen 1 (BPAG1) and envoplakin (30). While desmoplakin and BPAG1 are constituents of desmosome and hemidesomsome respectively, plectin is expressed more widely. They are mainly localized to intermediate filaments (IF) and filament attachment sites at the plasma membrane and they are recognized as versatile organizers of cytoskeletal architecture. The plakins are predicted to contain globular amino- and carboxyl-terminal domains that are separated by a central coiled-coil rod domain (31-36). The central rod domain is rich in heptad repeats and is believed to form a parallel α-helical coiled-coil with a dimerization partner or to assemble into higher-order filamentous structures with themselves or other coiled-coil proteins (30). Loss of plectin function, as in the case with BPAG1, has been linked to skin diseases such as epidermolysis bullosa possibly caused by disruption of anchorage site of keratin filaments to hemidesmosomes (37). On the other hand, abnormal expression of desmosomal proteins such as desmoplakin contributed to tumorigenicity and invasion in bladder and other cancers (38-40).

RESULTS AND EXPERIMENTAL METHODS

Specific aim 1: Define the ECM ligands that elicit normal breast and breast tumor phenotypes in a reconstituted basement membrane.

Murine mammary epithelial cell lines competent for milk protein expression were used to identify microenvironmental factors that regulate lactoferrin, a secreted iron binding protein which is expressed during normal functional development of mammary epithelium.

(A) Lactoferrin expression in mammary epithelial cells is mediated by changes in cell shape and actin cytoskeleton (see publication #2)

Induction of lactoferrin gene expression in cultured mouse mammary epithelial cells by laminin

Tissue-specific expression of milk protein genes in primary and secondary mouse mammary epithelial cells has previously been shown to depend on lactogenic hormones and contact with reconstituted basement membrane. To begin to determine the specific factors which influence lactoferrin (LTF) gene expression in mammary epithelium, the CID-9 mouse mammary epithelial cell line, was cultured in the absence or presence of reconstituted basement membrane (EHS matrix) with or without the mammotrophic hormones, prolactin. LTF message was not observed in total RNA harvested from medium density culture of CID-9 attached to tissue culture plastic, but was easily demonstrated in RNA from the same cells attached to EHS matrix. Unlike the

caseins, the expression of LTF was independent of prolactin. Further characterization of the matrix effect was performed with a clonal derivative of CID-9 cells, SCp2. Growing cultures of SCp2 cells, initially negative for LTF mRNA expression were deprived of fetal calf serum for 72 hours, then given 50 μg/ml of purified laminin, a major basement membrane component, or 5% fetal calf serum in the culture medium for an additional 6 or 48 hours prior to harvest. The cells exposed to laminin aggregated into clusters or round, refractile cells. In the culture exposed to fetal calf serum, the SCp2 cells achieved confluence by 48 hours. Incubation with either laminin or fetal calf serum was sufficient to induce LTF mRNA expression by 48 hours. This result was in striking contrast to β-casein mRNA which specifically required laminin for expression. The fetal calf serum result suggested that changes in cell-cell interactions, cell growth, and/or cell shape could replace the conditions for LTF induction provided by basement membrane.

Specific aim 2: Identify the specific integrins (ECM receptors) that transduce the ECM signal to the cells, by examining their expression in normal and tumor cells and elucidating their functional role by disrupting the signaling pathways.

(A) Cellular growth and survival are mediated by β1 integrins in normal human breast epithelium but not in breast carcinoma (see publication #5)

Formation of acinar structures within EHS is integrin dependent

To determine which of the various integrins expressed by human breast epithelial cells are functionally relevant to the formation of the acinar structures, specific inhibitory anti-integrin antibodies were used to interfere with this process in reconstituted basement membrane culture. Nonmalignant S1 cells cultured in EHS without antibodies, or with 10 μg /ml or 100 μg/ml of non-immune mouse or rat IgG, formed well-organized acinar structures at similar frequencies. In contrast, inhibitory anti-β1 subunit antibodies, at similar concentration, severely impaired the formation of spheres by S1 cells relative to control cultures. These effects were observed with two different anti-β1 antibodies and were dose dependent: mouse anti-β1 mAb (JB1a) induced a two-fold inhibition of sphere formation at 40 μg/ml and a 4-fold inhibition at 200 μg/ml, whereas rat anti-β1 mAb (AIIB2) caused an almost complete inhibition at 100 μg/ml. These data suggested that sphere formation by normal human mammary epithelial cells in response to EHS is dependent on integrin(s) of the β1 integrin family.

To define more precisely which integrins were critical in signaling acinar morphogenesis, similar experiments were performed with inhibitory anti-α2, -α3, and -α6 antibodies. Anti-α3 reduced sphere formation by 50% at 10 μg/ml and by 80% at 100 μg/ml. Anti-α5 and anti-α 6 antibodies were much less effective (about 70% of control) at both 10 μg/ml and 100 μg/ml, while inhibitory anti-α2 antibodies showed no appreciable inhibition. These data indicate that, of the integrin subunits assayed, α3β1 appears to be the most significant in mediating morphogenesis in a basement membrane matrix.

The inhibition of acinar morphogenesis by anti-integrin antibodies is associated with an inhibition of cell growth

Since normal mammary cells seeded into EHS in the presence of inhibitory anti-integrin antibodies remain suspended as single cells for the duration of the experiments, we asked whether the inhibitory antibodies interfered with acinar formation by blocking cellular growth. The thymidine-labeling indices (TLI) of S1 cells cultured in EHS in the presence or absence of inhibitory anti-integrin antibodies were measured. In the absence of inhibitory anti-β1 antibodies the TLIs were approximately 60% at day 2 and fell to 3% in EHS by day 6 as the cells formed differentiated acini. In contrast, in the presence of inhibitory antibodies, the cells remained suspended as single cells, and the TLIs were low at day 2 in both substrata and remained low throughout the experiment. At day 6 of culture in EHS, the low TLIs observed in the presence of anti-β1 antibodies reflect a small number of growth-arrested differentiated cells that formed acini by escaping the antibody blockade, and a majority of growth-inhibited single cells blocked by anti-integrin antibodies. These data suggest that an initial phase of cell growth is a requirement for acinar formation in three-dimensional culture. Thus, acinar formation appears to be a two-step process involving a β1-integrin-dependent cellular growth phase, followed by a phase of cell polarization to form the final organized structure.

Interruption of normal mammary cell-basement membrane interactions induces apoptosis

Prevention of appropriate cell-ECM contact by use of non-adhesive (polyHEMA) coated substrata, ECM fragments or RGD peptides can inhibit cell growth and differentiation in anchoroage-dependent cells (Hayman et al., 1985; Ingber, 1990) and trigger programmed cell death or apoptosis (Frisch and Francis, 1994) . Data presented herein show that inhibition of mammary cell attachment to basement membrane by ligation of β1 integrins blocks cellular growth and acinar formation. We therefore asked whether the inhibition of mammary cell-BM interaction induces apoptosis. S1 cells were embedded within EHS matrix in the presence or absence of function blocking anti-1 antibodies and assayed for evidence of apoptosis at day 2 and day 6 of culture by detection of FITC-digoxigenin nucleotide labeling of 3'OH DNA ends using the ApopTag in situ apoptosis detection kit.

In the absence of anti-β1 antibodies, S1 cells formed acinar structures as described above. Apoptotic nucleic were detected infrequently (0.74%) at day 2, whereas at day 6, 6.3% of single cells not incorporated into acini and 2.5% of individual cells within acini were stained, with the ApopTag reagents. In contrast, in the presence of anti-β1 antbodies, 20.9% of the cells contained nuclei stained by ApopTag reagents at day 2 and at day 6, 59.9% of the nuclei were labelled. Interestingly, a small number of acini (~6% of control) formed in the presence of inhibitory anti-β1 integrin. The cells within these acini did not contain nuclei stained with the ApopTag reagents. These data suggest strongly that β1 integrins transmit signals from ECM that are required for survival.

(B) Reversion of the Malignant Phenotype of Human Breast Cells in Three-Dimensional Culture and In Vivo by Integrin Blocking Antibodies (see publication #3)

Function Blocking β1-Integrin Antibodies Cause Dramatic Phenotypic Reversion of the T4-2 cells.

Since previous results showed that T4-2 cells had both a higher total level and an elevated ratio of cell surface β1- to β4-integrins, we wondered whether the aberrant malignant behavior may be a reflection of the changes in these integrins. Accordingly, we examined the consequences of treatment in 3-D with varying concentrations of a previously characterized rat monoclonal β1-integrin antibody (clone AIIB2) which has been shown to inhibit ligand binding (Werb et al., 1989). The antibody caused massive apoptosis in S1 cells (), while T4-2 cells were refractory. Remarkably however, in addition to resistance to apoptosis, almost all the antibody-treated T4-2 tumor cells assumed a morphology which was indistinguishable from that observed S-1 cultures and was discernible as early as 4 d after incubation. To determine whether the antibody-treated T4-2 cells have truly reverted to a "nonmalignant" phenotype, we cryosectioned the colonies and examined their morphology by immunofluorescence confocal microscopy. As markers of normal acinar formation, we examined both cytoskeletal organization and superimposition and distribution of cadherins and catenins. Sections of S-1 acini revealed uniform and polarized nuclei (stained with propidium iodide; red), well-organized filamentous actin (FITC phalloidin; green), and uniformly superimposed E-cadherin and ß-catenin at the lateral cell-cell junctions. In contrast, untreated or IgG-treated tumor cells had polymorphic nuclei and a grossly disorganized actin cytoskeleton, visualized as random, hatched bundles. Additionally, E-cadherin and ß-catenin were not colocalized. In contrast, ß1-treated T4-2 cells revealed striking rearrangements of cytoarchitecture as demonstrated by their well-organized acini, and cytokeratin 18 intermediate filament etworks. Furthermore, organized adherens junctions became evident in T4-ß1 acini and were accompanied by the re-establishment of E-cadherin-catenin complexes. These changes were shown to occur in greater than 95% of the tumor colonies treated with blocking antibody, as quantified by analyzing the numbers of disorganized vs organized spheroids in relation to the S-1 and the mock-treated T4-2 cells.

To investigate whether the described phenotypic reversion is associated with cell cycle regulation, markers of proliferation and cell cycle status was examined in T4-ß1 cells. These cells showed a decrease in [^{3}H]thymidine incorporation and the size of the acini which was now composed of only 6-8 cells, similar to that observed for S-1 cells. Cryosections of T4-b1 colonies incubated with antibodies against either collagenIV or laminin revealed deposition of a basally distributed, almost continuous basement membrane, with characteristics similar to that observed in the S-1 acini. In contrast, punctate and inversely polarized collagen IV and laminin immunostaining were observed in the mock-treated tumor colonies. Thus, these tumor cells had retained the ability to deposit a basement membrane and to form polarized structures if the correct structural cues could be received. T4-ß1 colonies also had dramatically decreased cyclinD-1 levels again comparable to that seen in S-1 cultures and markedly reduced Ki-67 levels. In addition, T4-ß1 cells revealed a

drastic increase in the negative regulator of cell cycle p21. From these results, it is suggested that most reverted cells had exited the cell cycle and therefore had a reduced propensity to proliferate.

Reduced tumorigenicity of ß1-inhibitory antibody treated T4-2 cells in nude mice

To find out whether phenotypic reversion of tumor cells would be sufficient to reduce tumorigenicity in vivo, we injected tumor cells treated in suspension with ß1-integrin blocking mAb, mock mAb, or no treatment for 3 h, as well as S-1 cells into nude mice. Within two weeks small nodules were observed in all injected sites including the S-1 controls. Whereas these nodules regressed rapidly in the S-1 and T4-ß1 groups, actively growing tumors were observed in greater than 75-90% of the mock mAb or vehicle-treated T4-2 mice. Upon sacrifice we observed both a significantly reduced tumor number and tumor size in the T4-ß1 group. These data suggested that "normalization" of the tumor cell phenotype in culture has a counterpart in vivo where the malignant potential is reduced or lost.

Specific aim 3: Study the potential role of tumor suppressor genes in the regulation of breast cell-ECM interaction by transfecting these genes into breast tumor cells and assessing the consequence of expression on tumor cell growth and differentiation.

(A) Study of the role of a putative metastasis suppressor gene nm23-H1 in mammary development and differentiation (see publication #7)

Overexpression of nm23-H1 in human breast carcinoma cells leads to formation of basement membrane, production and apical secretion of sialomucins and growth arrest

To determine the effect of nm23-H1 gene expression on the morphological differentiation of breast cells, MDA-MB-435 breast carcinoma clonal cell lines transfected with pCMVBamneo vectors (C-100 and C-103) or the same vector containing the full length nm23-H1 cDNA (H1-170 and H1-177) were cultured within an EHS matrix for 12 days. Immunohistochemical staining of type IV collagen and laminin was conducted on parental MDA-MB-435 cells, control transfectants, and nm23-H1 gene transfectants cultured within an EHS matrix. By day 6 of culture, 87.8%± 3.8% of Nm23 protein-positive transfectants deposited type IV collagen; this percentage increased to 97.1% ±1.9% by day 12 of culture. In contrast, none of the parental cells, control transfectants, or Nm23 protein-negative H1-170 transfectants expressed type IV collagen or laminin. These results indicated that overexpression of nm23-H1 in MDA-MB-435 breast carcinoma clonal cell lines led to formation of endogenous basement membrane as seen in normal breast epithelial cells HMT-3522. In addition to basement membrane synthesis, 49.5%±4.5% of Nm23 protein-positive transfectants expressed sialomucin (a mammary differentiation marker) compared with 5.5%±1.5% of control.

To determine whether differentiation induced by ectopic expression of nm23-H1 was linked to signaling in growth arrest, cell number per colony and thymidine labeling indices were measured for C-100, C-103, H1-170, and H1-177 cell lines grown within an EHS matrix for 12 days. Nm23

protein-negative colonies contained a mean of 27 cells, while Nm23 protein-positive colonies contained a mean of 8.2 cells. The percentage of spheres that were basement membrane-positive-[^{3}H] thymidine negative rose from 7.6% on day of culture to 70.1% on day 12. A concurrent decrease in the percentage of basement membrane-positive-[^{3}H] thymidine-positive spheres was observed from 80.2% on day 6 to 27.0% on day 12. These data suggested the hypothesis that basement membrane synthesis and secretion, an early event in the 3D matrigel culture system, may signal an inhibition of cell growth.

To further address the specific aim #3, we have used differential display to isolate novel breast tumor suppressors. In the 1996-1997 annual progress report, we have described characterization of a putative tumor suppressor gene AZ-1 isolated by comparing message levels of two phenotypically and functionally distinct cell lines [premalignant (S2) and tumor (T4-2) cells] of the HMT3522 breast cancer progression series. In accord with the differential expression patterns shown by RT-PCR differential display , AZ-1 was found to be highly expressed in S2 cells and the levels were significantly lowered in T4-2 cells by northern analysis. The 4.4-kb message size of AZ1 was also observed in several nonmalignant breast cells including nonmalignant (S1), MCF10A, and primary luminal epithelial cells from mammoplasties. In contrast, AZ-1 message was low or not detected in ten breast epithelial cell lines examined. Moreover, in comparison with the level in normal breast tissues AZ-1 message was low or absent in all four breast carcinoma biopsies.

Sequence analysis of AZ-1 indicates that it encodes a protein size of 64 kD. Secondary structure prediction of the AZ-1 protein showed that it contains an extended coiled-coil domain at its C-terminus and an upstream repressor LexA-like HTH motif. BLAST (Basic Local Alignment Search Tool) search results indicated that the putative full-length 471 amino acid of AZ-1 coding sequence did not match any known gene. However, the coiled-coil domain of AZ-1 protein sequence showed limited homology with the α-helical rod region of plakin family proteins including plectin, desmoplakin and envoplakin. In addition, the distal residues of AZ-1 coiled-coil region contain sequences similar to the intermediate filament (IF) signature LEF motif.

Based on the differential expression patterns shown by northern analyses, it is plausible that AZ-1 gene could encode a breast tumor suppressor. We have tested this hypothesis by transfecting AZ-1 into T4-2 cells and examined the functional and phenotypic changes of the AZ-1 stable transfectants.

(B) Functional analysis of the putative tumor suppressor gene AZ-1 in the breast tumor development (publication#1)

AZ-1 gene is re-expressed in phenotypically-reverted tumor cells

Since AZ-1 is homologous to myosin heavy chain and plakin family proteins that are know to be involved in organization of cytoskeletal architecture, we were interested in determining whether AZ-1 gene expression could be modulated by phenotypic alteration of tumor cells in a reversion

system described previously. Briefly, when T4 cells were cultured in the presence of inhibitory ß1-integrin antibody in a 3-D assay system, they reverted morphologically to S1-like cells. They formed acini while re-assembled a basement membrane, re-organized cytoskeletal network, suppressed cyclin D1 and were growth arrested. Interestingly, AZ-1 gene was up regulated in the reverted T4 cells (analogous to p21) to a level reminiscent of that seen in the S1 cells. The recurrence of AZ-1 in normalized breast tumor cells could be linked to its putative function in cytoskeletal reorganization. However, further studies will need to confirm this hypothesis.

Ectopically-expressed AZ-1 in human breast tumor cells (T4-2) leads to normalization of cell morphology, reduced invasiveness and anchorage-independent growth

The functional roles of AZ-1 in breast tumor progression were examined by transfecting a retroviral expression construct (pAZ1-LXSN) containing the full-length AZ-1 cDNA into T4-2 cells. T4-2 cells stably transfected with pLXSN and pAZ1-LXSN constructs were grown in a three-dimensional reconstituted basement membrane (EHS) culture. Cells transfected with a control pLAPSN construct encoding an alkaline phosphatase were also analyzed. Similar to the untransfected T4-2 cells, at day 10 both pLXSN and pLAPSN transfected cells outgrew to form large, disorganized colonies (average colony size 175 mm). In sharp contrast, pAZ1-LXSN transfected cells formed normal size spheroids (average colony size 40 mm) with more organized cytoskeletal organization reminiscent to that of the nonmalignant (S1) cells. Tumor suppression function of AZ1 was further examined by a matrigel invasion assay. In comparison with vector transfected T4-2 cells, ectopically expressed AZ1 drastically reduced the invasion potential of the breast tumor cells by 70-85%. The clonogenicity (also known as anchorage-independent growth) of pAZ1-LXSN transfected T4-2 cells determined by a soft agar assay was only 10-20% of the vector controls. These data suggested when assayed in an *in vitro* system ectopically-expressed AZ-1 reverted the tumor cells to a more normal-like morphology and a less tumorigenic phenotype.

The in situ tumor suppressor role of AZ-1 was also examined by injecting the AZ-1 stable transfectants into nude mice. The preliminary results are encouraging and repeated experiments are in progress and the final results will be reported in the manuscript in preparation.

CONCLUSIONS

We have provided a complete list of the accomplishments that addressed the three proposed specific aims in this final report for the grant titled "Mechanisms of Abnormal Cell-Matrix Interactions in Human Breast Cancer." The initial phase (1994-1995 and the early part of 1995-1996) of the research efforts were conducted under the supervision of Drs. Mina Bissell and Anthony Howlett, the former principal investigator of the grant. During this period, the investigation was focused on understanding the aberrant cell-extracellular matrix (ECM) interactions in breast tumor cells. One of the major findings was the functional and phenotypic reversion of the breast tumor cells cultured in a three-dimensional system by an inhibitory anti-β1

integrin antibody (AIIB2). Further studies are underway to understand the molecular mechanisms involved and to explore the clinical implication of the β1 integrin reversion system.

The second phase of the research was conducted by Dr. Huei-Mei Chen, the designated principal investigator of the grant. One of the exciting findings under Dr. Chen's supervision is the isolation of a putative tumor suppressor gene AZ-1. AZ-1 protein has a coiled-coil domain that shows homology to the dimerization domain of the plakin family proteins including desmoplakin. One of the interesting functions of AZ-1 revealed by the *in vitro* matrigel invasion assay was the demonstrated "invasion suppression" capability. The tumor suppressor function of AZ-1 was also examined in an *in vivo* system and the preliminary results were encouraging. Recent attempts on the biochemical characterization of AZ-1 protein by immunoprecipitation with anti-AZ-1 antibody showed AZ-1 may be involved in the intricate cell adhesion network and further studies are underway to extend our understanding of molecular mechanisms underlying the functions of this newly-discovered novel tumor suppressor candidate gene.

Research findings reported here outline only the beginning of the committed long-term efforts set forth to uncover the mysteries involved in the breast tumor progression. We will continue to unravel breast cancer relevant genes by the differential display protocol employed in this study and to explore functions of these genes in the established breast cell culture and *in vivo* systems.

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KEY RESEARCH ACCOMPLISHMENTS

1. Isolated and sequence mapping of two novel tumor suppressor candidate genes, AZU-1 and MHFK by comparing gene messages of premalignant and tumor breast epithelial cells

2. Demonstrated tumor suppressor function of AZU-1 gene by in vitro and in vivo tumorigenicity assays

3. Reexpressed AZU-1 in breast tumor cells could revert tumorigenic phenotype

4. Used a functional three-dimensional basement membrane culture system and demonstrated that a functional blocking β1-integrin antibody reverted the tumorigenic phenotype

APPENDICES

1. LIST OF PUBLICATIONS AND MEETING ABSTRACTS

Publications:

1. Chen HM, Schmeichel KL, Mian IS, Petersen OW, and Bissell MJ. 1999. AZU-1: a candidate breast tumor suppressor and biomarker for tumorigenic reversion. Accepted for publication in Mol. Biol. Of the Cell.

2. Close MJ, Howlett AR, Roskelley CD, Desprez PY, Bailey N, Rowning B, Teng CT, Stampfer MR, and Yaswen P. Lactoferrin expression in mammary epithelial cells is mediated by changes in cell shape and actin cytoskeleton. J. Cell Sci. 1997,110:2861-2871.

3. Weaver VM, Petersen OW, Wang F, Larabell CA, Briand P, Damsky C, and Bissell MJ. Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies. J Cell Biol 1997, 137:231-245.

4. Chen HM, Weaver VM, Petersen OW, and Bissell MJ. Extracellular matrix (ECM) as a central regulator of function, growth and programmed cell death in breast cells of both mice and men: implications for therapy. 1996, The Pezcoller Foundation Journal 3, p.7-11.

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PATENTS

Chen, H.M. and M.J. Bissell. 1999. Human AZU-1 gene, variants thereof and expressed gene products. PVJB Ref.: 2960.44 (HV), U.S. Serial No. 09/344,624, U.S. and international patent application filed on 6/25/99, 69 pp.

Chen, H.M. and M.J. Bissell. 1999. Isolation and functional characterization of a novel breast tumor suppressor candidate gene MHFK (MHFK1 and MHFK2). PVJB Ref. 2960.43 (HV), provisional patent application filed on 7/15/99.

Meeting Abstracts:

1. Chen HM, Schmeichel KS, Mian IS, Lelievre S, Petersen OW and Bissell MJ. 1999. AZU-1: a candidate breast tumor suppressor and biomarker for tumorigenic reversion. Abstract submitted for American Society for Cell Biology Meeting. Dec 11-15. Washington DC.

2. Chen HM, Petersen OW, and Bissell MJ. 1998. Up-regulation of a novel breast tumor suppressor candidate gene AZ1 correlates with tumorigenic reversion and cytoskeletal reorganization. Abstract submitted for American Society for Cell Biology Meeting. Dec 13-18. San Francisco, CA.

3. Chen HM, Weaver VM, Wang F, Petersen OW, and Bissell MJ. 1997. A novel gene lost when human breast cells become malignant could serve as a progression marker. Abstract submitted for DOD Era of Hope meeting. Oct.31- Nov.4, Washington DC.

4. Chen HM, Petersen OW, and Bissell MJ. 1997. A novel gene lost when human breast cells become malignant is reexpressed in reverted cells. Abstract submitted for American Society for Cell Biology meeting meeting, Dec.13-17, Washington DC.

5. Weaver VM, Clark S, Petersen OW, and Bissell MJ. 1995. Refractoriness to β1-integrin antibody-induced apoptosis precedes malignant transformation in HMT-3522 mammary epithelial cells: a culture model of progressive human breast cancer. Abstract submitted for American Society for Cell Biology meeting, Dec. 10-14.Washington DC.

INVITED SEMINARS

Department of Defense Breast Cancer Research Program's Era of Hope Meeting. "A Novel Gene Lost When Human Breast Cells Become Malignant is Reexpressed in Reverted Cells," Washington DC, Oct. 31 - Nov. 4, 1997.

Chiron Corporation. "Application of a Unique Breast Cancer Progression Model for the Study of Tumorigenic Reversion and Gene Discovery," Emeryville, CA, July 16, 1998.

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Weaver VM (postdoctoral fellow)
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ADDENDUM (Publication #1)

AZU-1: a candidate breast tumor suppressor and biomarker for tumorigenic reversion

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Running title: AZU-1 is a candidate tumor suppressor

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Abbreviations used: AZU-1, anti-zuai-1; CCD, coiled-coil domain; EGFR, epidermal growth factor receptor;SPAZ domain, Serine-Proline rich AZU-1 domain; 3D rBM, 3-Dimensional reconstituted basement membrane; TACC, transforming acidic coiled coil.

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ABSTRACT

To identify genes misregulated in the final stages of breast carcinogenesis, we performed differential display to compare the gene expression patterns of the human tumorigenic mammary epithelial cells, HMT-3522-T4-2, with that of their immediate pre-malignant progenitors, HMT-3522-S2. We identified a novel gene, called AZU-1, that was abundantly expressed in non- and pre-malignant cells and tissues but was appreciably reduced in breast tumor cell types and in primary tumors. The AZU-1 gene encodes an acidic 571 amino acid protein containing at least two structurally distinct domains with potential protein-binding functions: an N-terminal serine and proline-rich domain with a predicted Ig-like fold and a C-terminal coiled-coil domain. Re-expression of AZU-1 in T4-2 cells was sufficient to reduce their malignant phenotype substantially, both in culture and *in vivo*. Reversion of the tumorigenic phenotype of T4-2 cells, by other means described previously, also was accompanied by the re-expression of AZU-1. These results indicate that AZU-1 is a candidate breast tumor suppressor that may exert its effects by promoting correct tissue morphogenesis.

INTRODUCTION

Significant advances in breast cancer research have been gained from studies of disease-linked genetic mutations. The identification of genes such as BRCA-1 and BRCA-2 confirms that inherited genetic lesions can influence tumorigenic conversion of breast epithelial cells, either by activating oncogenes or inactivating tumor suppressors (Haber and Harlow, 1997; Sager, 1997). Increasingly, studies indicate that, along with predisposing chromosomal abnormalities, misexpression of genes with otherwise wild-type sequences, also contributes to the process of tumorigenesis (Zhang et al., 1998). For example, the growth factor receptors ErbB1 and ErbB2, which are overexpressed in breast tumor tissue *in vivo*, have become accepted prognostic indicators for breast cancer diagnosis, and therapies aimed at reducing their levels are now in clinical trials (Pinkas-Kramarski et al., 1997). Thus, comparisons of gene expression patterns in normal and tumor cells is a promising strategy for discovering gene function and for eventually understanding, diagnosing and treating cancer of the breast.

The results of comparative gene expression studies, while continuing to demonstrate the importance of growth regulators and transcription factors in cancer progression, have also implicated other cancer-related genes with surprisingly diverse functions. In the case of breast cancer, these include proteases and protease inhibitors (Zou et al., 1994; Sternlicht et al., 1999), extracellular matrix components (Zhang et al., 1998) and cytoskeletal elements (Sager, 1997; Mielnicki et al., 1999). Such gene misregulation can be due to defects in the breast epithelial cells themselves, or can be due to the effects of neighboring cells, such as myoepithelial or stromal cells, that could indirectly influence the behavior of the epithelial cells (Zou et al., 1994; Lochter et al., 1997).

A recently developed human epithelial breast cell model, the HMT-3522 progression series, originated from primary breast epithelial cells of a woman diagnosed with fibrocystic breast disease. Serial culture of these cells allowed for the generation of a continuum of genetically-related cell populations that range in phenotype from non-malignant (S1) to pre-malignant (S2) to tumorigenic (T4-2) (Briand et al., 1987; Briand et al., 1996). Because these cell lines share common genetic origins, observed differences in gene expression patterns between these cells are likely indicative of changes that influence tumorigenic progression rather than differences in

genetic backgrounds.

To identify genes that were misexpressed upon tumorigenic conversion in the breast, we used a differential display strategy to compare the gene expression profiles of tumorigenic T4-2 cells with their pre-malignant S2 progenitors. Here, we report the identification and characterization of a novel gene we refer to as AZU-1, that is expressed abundantly in non-malignant (both primary and immortalized) and pre-malignant breast epithelial cells, but is dramatically down-regulated in a number of breast tumor cell lines and primary tumors. Restoration of normal AZU-1 expression levels in T4-2 cells was sufficient to reduce tumor formation *in vivo* and resulted in phenotypic reversion in culture (Weaver et al., 1997). Collectively, our results suggest that AZU-1 may protect non-malignant cells from tumorigenic conversion by promoting proper cellular organization and tissue morphogenesis.

MATERIALS AND METHODS

Cell culture

HMT-3522 human mammary epithelial cells (S1, S2 and T4-2) and MCF10A cells were grown in chemically-defined medium (Briand et al., 1987; Soule et al., 1990; Briand et al., 1996). HMT-3909 and MCF-7 cells were cultured on type I collagen-coated dishes in DMEM/F12 medium supplemented with 1.4 X 10^{-6} M hydrocortisone and 2 mM glutamine, respectively. Primary human breast epithelial cells were purified and cultured as previously described (Petersen and van Deurs, 1987). Protein extracts were prepared from monolayer cultures using established protocols (Wang et al., 1998).

3-Dimensional reconstituted basement membrane (3D rBM) cultures were generated as described previously (Petersen et al., 1992; Weaver et al., 1997) using a commercially prepared rBM (Matrigel; Collaborative Research, Waltham, MA). 3D rBM assays were evaluated by phase contrast microscopy and by measuring colony diameter using an eyepiece equipped with a micrometer spindle. Cellular polarity was determined by immunostaining for the basal markers collagen IV and b4 integrin (Weaver et al., 1997). Reversion assays, using the b1 integrin function-blocking antibody mAb AIIB2 and Tyrphostin AG 1478 (Calbiochem, San Diego, CA), were performed as described earlier (Weaver et al., 1997; Wang et al., 1998).

RNA extraction and northern blot analysis

Total RNA was extracted from cells and tissues using TRIzol reagent (Life Technologies, Inc. Grand Island, NY). For northern blots, total RNA (20 mg/lane) was resolved on denaturing agarose gels and transferred to Hybond-N^+ membranes (Amersham; Cleveland, OH). Resulting blots were hybridized with ^{32}P- labeled cDNA probes and analyzed by autoradiography. A GAPDH probe was used to control for sample loading. Relative band intensities were quantified by densitometric analysis.

Differential display

Differential display was performed using the RNAimage kit as per manufacturer's instructions (GenHunter Corp., Nashville, TN). Briefly, total RNA (DNA-free) from S2 and T4-2 cells was reverse-transcribed and the cDNA products were amplified by polymerase chain reaction using the anchored (H-T_{11}M, M=A,C,G) and arbitrary (H-AP-1) primers provided in the kit and a[^{33}P]dATP. PCR products were resolved on denaturing gels and differential expression was evaluated by autoradiography. Confirmation of the expression pattern of a 180 bp cDNA was

achieved by subjecting the fragment to a second PCR amplification and by analyzing the products on agarose gels.

AZU-1 cloning strategy

The sequence of the 180 bp differential display cDNA fragment was compared to existing Genbank sequences and was found to be identical to three ESTs (*Homo sapiens* cDNA clones N57107, R38679 and H23488). All three clones contained the 180 bp plus additional 5' and/or 3' sequences. Two of these clones exhibited polyadenylation sites, and none displayed apparent open reading frames. Rapid amplification of cDNA ends (5' RACE; Life Technologies) was performed to characterize the 5' sequence of the identified gene. Primers corresponding to the 180 bp differential display fragment were used to initiate the 5' RACE procedure according to manufacturer's instructions. The protocol was repeated 12 times to obtain 3.8 kb of sequence; in each cycle, 500-800 bp of additional 5' sequence was obtained. Sequencing was conducted using cycle sequencing (Amersham Life Science, Cleveland, OH). The 3.8 kb sequence contained a candidate translation start codon (consistent with the Kozak consensus rules) (Kozak, 1984) and a downstream in-frame stop codon.

To confirm the accuracy of the 3.8 kb AZU-1 sequence, and to generate a composite AZU-1 cDNA, primers corresponding to AZU-1's 5' and 3' ends were used in polymerase chain reactions. In two independent experiments, each using a distinct pool of total S1 cellular cDNA as a template, the resulting PCR products were identical in composition to the sequence obtained using 5' RACE. We call the isolated gene AZU-1 (Genbank accession number AF176646). Full-length AZU-1 cDNAs were subcloned into pCR 2.1 (pCR2.1-AZU-1; InVitrogen, Carlsbad, CA) for further amplification and use. The pI of AZU-1 was determined using GCG software (Madison, WI).

AZU-1 constructs

To subclone AZU-1 coding sequences into pET-28a (Novagen, Madison, WI), PCR was performed using pCR2.1-AZU-1 as a template and primers supplemented with SacI and SalI restriction sites (forward primer = $^{5'}$-CTGAGCTCATGCCCCTGAGGAGGCCAAAGAT-$^{3\phi}$; reverse primer = $^{5'}$-GCGTCGACTTTAGCTTTTCCCCATTTTGGCAATCAGTTC-$^{3\phi}$). pCIneo-AZU-1 and pLXSN-AZU-1 constructs were generated by subcloning NheI/XhoI and EcoRI/XhoI cDNA fragments from pET-28a-AZU-1 into pCIneo (Promega, Madison, WI) and pLXSN (Clontech, PaloAlto, CA), respectively.

In vitro transcription/translation

In vitro transcription/translation reactions were performed using the TNT coupled reticulocyte lysate kit (Promega) as per manufacturer's instructions. Luciferase cDNA (mw = 61 kDa) was used as a positive control. ^{35}S-labeled AZU-1 produced in the *in vitro* transcription/translation was immunoprecipitated in RIPA buffer in the presence of 1 ml whole rabbit serum, either pre-immune or AZU-1-specific, as described previously (Weaver et al., 1997). Molecular mass of AZU-1 was determined using ChemiImager software (Alpha Innotech, San Leandro, CA).

AZU-1 antibody production and western immunoblots

A polyclonal antibody was generated against a 20 amino acid N-terminal AZU-1 peptide supplemented with a C-terminal cysteine and coupled to a protein carrier (MPLRRPKMKKTPEKLDNTPAC; ImmunoVision Technologies, Daly City, CA). Pre-immune

and immune sera were used as probes in western blots at a dilution of 1:250. Primary antibody binding was detected using an HRP-conjugated goat anti-rabbit secondary antibody followed by chemiluminescent detection.

Re-expressing AZU-1 by retroviral infection

AZU-1 re-expression in T4-2 cells was achieved using the Retro-X viral gene delivery system (Clontech) according to manufacturer's protocols. The studies performed here were done on pooled populations of T4-2 cells that were stably-infected with the vector alone (pLXSN) or with AZU-1 sequences (pLXSN-AZU-1). The AZU-1 transgene comigrates with the endogenous AZU-1 message at 4.4 kb. Northern blots probed with sequences from AZU-1's 3' UTR show no increase in endogenous AZU-1 expression in AZU-1-overexpressing cells. Thus, the increased AZU-1 expression observed in the T4-2-AZU-1 cells was entirely attributable to expression from the AZU-1 transgene (not shown).

Assays of tumor phenotype

For soft agar assays, cells were seeded at 1 X 10^5 cells/well in 0.35 % soft agar in 12-well plates. After 4 weeks, colonies greater than 40 mm were scored as positive for growth (Wang et al., 1998). Invasion assays were performed as described previously (Lochter et al., 1997). The data are expressed as the number of cells per field at 200X magnification. Tumorigenic potential was assessed by subcutaneous injection of 2.5 X 10^6 cells into flanks of 4-6 week old BalbC nu/nu female mice. Tumor nodules were measured using a caliper six to eight weeks after injection.

RESULTS

Identifying putative determinants of tumorigenic conversion by differential display

We utilized a PCR-based differential display strategy to screen for genes that were variably expressed in S2 and T4-2 cells. We detected a 180 bp cDNA that was present at higher levels in the S2 cells than in their T4-2 counterparts. The cDNA fragment was isolated, amplified and used as a probe in northern blots of total RNA from these cells. In S2 cells, the probe hybridized with an abundant 4.4 kb message, and two minor transcripts of approximately 7.5 and 9.5 kb (Figure 1A). The T4-2 cells displayed a dramatic reduction in the expression of the 4.4 kb message in comparison with S2 cells.

Northerns using probes derived from the coding sequences (see below) confirmed the expression pattern of the 4.4 kb gene product. We detected an abundant and specific message not only in the non-malignant human epithelial cell lines, HMT-3522-S1 and MCF10A, but also in primary cultures of human luminal epithelial and myoepithelial cells (Figure 1B). Expression of the 4.4 kb message was significantly reduced in 10 of the 11 breast carcinoma cell lines examined (Figure 1C). Likewise, two of three carcinomas showed reduced AZU-1 expression when compared to normal tissue (Figure 1D). Based on these observations, and the functional studies described below, we have named this gene product anti-zuai-1 (or AZU-1), with "zuai" meaning "breast cancer" in Chinese.

AZU-1 protein expression and sequence analysis

We used 5' RACE to recover a full-length AZU-1 cDNA and found that the AZU-1 sequence did not correspond to any previously published gene. The AZU-1 encodes a protein of 571 amino acids with an estimated pI of 5.1 (Figure 2A). Although predicted to be 64 kDa, the full-length AZU-1 protein, when produced *in vitro*, displays a significantly higher relative mobility of 80 kDa when resolved on denaturing gels (Figure 3A). An AZU-1-specific antibody recognized both the *in vitro*-translated AZU-1 protein (Figure 3A) and a protein of identical size in HMT-3522 cell extracts (Figure 3B). Like the transcript, AZU-1 protein levels were significantly reduced in T4-2 cells; on average, AZU-1 protein levels were three-fold reduced in T4-2 cells in comparison to their non-malignant S1 counterparts (mean: 3.0 ± .85, n=11).

Using BLAST analysis (Altschul et al., 1997), we found that AZU-1 shares significant similarity (particularly at its N- and C-termini) with three sequences deposited in Genbank, called TACC1 (Still et al., 1999), TACC2 and TACC3 (Still et al., 1999) (TACC = transforming acidic coiled coil; Genbank loci AF049910, AF095791 and AAD25964, respectively). TACC2 is most similar to AZU-1 and is likely to be an AZU-1 splice variant since, apart from two small insertions and a single amino acid change, it is identical to AZU-1 at both the nucleic acid and protein levels. The second most closely related gene to AZU-1 is TACC1, a gene cloned from the breast cancer amplicon 8p11 (Still et al., 1999). TACC3, although more distantly related to AZU-1 than TACC1, is also similar to AZU-1 with respect to both its domain organization and amino acid sequence. These genes may thus represent a new superfamily.

Alignment of AZU-1 with TACC1 and TACC3 suggests four AZU-1 protein domains (Figure 2B). At its N-terminus, AZU-1 exhibits a domain of 83 amino acids that we call a "SPAZ" domain (for Serine- and Proline-rich AZU-1 domain; Figure 2C). The combined serine-proline content of this domain is 36%. SPAZ domains are found in AZU-1 (or TACC2), TACC1, TACC3 and the *S. cerevisiae* gene product BCK1, a member of the MAPKKK family of serine/threonine kinases (Lee and Levin, 1992). In all of these gene products, two serine residues in the domain are invariant.

AZU-1's central domains, called Region I and Region II, are defined by virtue of their relationship to TACC1 (multiple sequence alignment not shown). Region I shows some sequence identity (20%) with the corresponding region of TACC1. One particular sequence motif common to both AZU-1 and TACC1 in Region I (HATDEEKLA; highlighted in Figure 2A) is not conserved in TACC3. Region II corresponds to the segment in AZU-1 that is absent from TACC1 (and present only partially in TACC3). PSORT predictions (Nakai and Horton, 1999) indicate that AZU-1 contains two putative nuclear localization sequences (NLSs), one at its N-terminus and one at amino acid 122 (Figure 2A).

The fourth and C-terminal region of AZU-1 displays a series of heptad repeats consistent with the presence of an extensive, but discontinuous, coiled-coil domain (CCD, Figure 2D). The seven structural positions of each heptad repeat are named a-g; positions a and d (capital letters in Figure 2D) are occupied by hydrophobic residues and are predicted to form a non-polar helix interface whereas the remaining residues are hydrophilic and form the solvent-exposed part of the helix surface (Lupas, 1996; Lupas, 1997).

While most homologous to TACC1 and TACC3, AZU-1's coiled-coil domain is also similar to that of the human SB1.8/DXS423E protein, a putative homolog of the *S. cerevisiae* SMC1 protein that is essential for proper chromosomal segregation during mitosis (PIR locus I54383) (Rocques et al., 1995). Alignments indicate three major regions where the characteristic heptad repeats of the CCD fall into register in all four proteins (Figure 2D). The Multicoil program predicts that all of these domains are likely to form dimers (probability > 0.90) (Wolf et al., 1997).

Assays of AZU-1 tumor suppressor function in vivo and in culture

Reduced expression of AZU-1 in a high percentage of tumorigenic cell lines suggested that the loss of AZU-1 may play a role in tumorigenic conversion. To test this hypothesis, we asked whether re-expression of AZU-1 in T4-2 cells is sufficient to attenuate their tumorigenic phenotype. Using a viral-mediated gene transfer system, we introduced a full-length AZU-1 transgene into T4-2 cells. Pooled populations of stably-infected cells were screened for AZU-1 expression and were shown to contain AZU-1 message and protein levels comparable to those observed in S1 cells (Figure 4A and B). These levels were approximately 2- to 3-fold higher than AZU-1 expression in the vector-infected T4-2 cells.

To test the potential tumor suppressor function of the AZU-1 gene product, assays of anchorage-independent growth and invasive potential were performed (Figure 4C and D, respectively). S1 and T4-2 cells displayed expected behaviors in these assays: S1 cells did not support growth in soft agar and were non-invasive, while T4-2 cells (uninfected or vector-infected) gave positive responses in both assays. T4-2-AZU-1 cells showed a significantly diminished tumor phenotype in soft agar and invasion assays, with behavioral responses that were 25% (for soft agar assays) and 15% (for invasion assays) of those displayed by the vector-infected T4-2 cells.

S1 cells and T4-2 cells and their corresponding AZU-1 transfectants were also examined for tumorigenicity *in vivo* (Table I). As reported previously (Briand et al., 1987; Briand et al., 1996; Weaver et al., 1997), S1 cells failed to give rise to tumors when injected into nude mice, while the T4-2 cells produced tumors in about 90% of the injected sites. Mice injected with T4-2-AZU-1 cells gave a significantly reduced tumorigenic response with only four of the 32 inoculated sites (13%) producing detectable tumors; these tumors were approximately 7-fold smaller than those formed by control T4-2 cells (Table 1).

Restored AZU-1 levels promote normal tissue architecture in tumorigenic breast cells in culture

We demonstrated previously that normal and tumorigenic breast cell phenotypes can be effectively distinguished in the context of 3-dimensional reconstituted basement membrane (3D rBM) assays (Petersen et al., 1992; Weaver et al., 1997). In 3D rBM assays, S1 cells, embedded in a laminin-rich matrix, form polarized, growth-arrested, acinar structures characterized by polarized b4 integrin localization and basal deposition of endogenous BM (Collagen IV). T4-2 cells, cultured under the same conditions, form large, growing and unpolarized colonies with higher, but disorganized, b4 integrin and Collagen IV deposition. In the presence of inhibitors of b1 integrin or EGFR, T4-2 cells undergo "phenotypic reversion" to form near-normal growth-arrested acini similar to those formed by S1 cells (Weaver et al., 1997; Wang et al., 1998). Thus, culturing cells in 3D rBM provides a simple, yet informative, assay that allows for the evaluation of tissue polarity and architecture as well as cellular growth.

We asked whether re-expression of AZU-1 would be sufficient to cause phenotypic reversion of T4-2 cells in the 3D rBM assay. AZU-1-overexpressing-T4-2 and control cells were embedded in 3D rBM gels. As expected, after 10 days, S1 cells formed small, uniform, multicellular spheres with organized basement membranes and basally-localized b4 integrin (Figure 5A and ref. (Weaver et al., 1997)). T4-2 colonies (both unmodified and vector-infected) continued to grow and formed large, irregular and unpolarized colonies (Figure 5A). In contrast, T4-2-AZU-1 cells underwent phenotypic reversion, forming S1-like colonies displaying appropriate cellular polarity. These results indicate that re-expression of AZU-1 at levels comparable to non-malignant cells is sufficient not only to reduce the growth capacity of the tumor colonies, but also to facilitate the structural reorganization required to produce polarized, organotypic structures.

Phenotypic reversion of T4-2 cells requires bi-directional cross-talk between at least two signalling pathways, b1 integrin and EGFR, (Wang et al., 1998). We showed previously that inhibition of either pathway reduced the signalling activity of the other and resulted in the reduction of total b1 integrin and EGFR protein levels. Given the ability of AZU-1 to revert the T4-2 phenotype, we reasoned that its gene product might also be part of the orchestrated signalling events. If so, its expression might be expected to be up-regulated during b1 integrin/EGFR-dependent reversion. To test this hypothesis, we measured the AZU-1 mRNA levels in T4-2 cells treated with or without inhibitors of either b1 integrin (mAb AIIB2) or EGFR (tyrphostin AG1478) (Figure 5B, panel a). In comparison with untreated T4-2 cells, AZU-1 expression was significantly higher in T4-2 cultures treated with the b1 integrin or EGFR antagonist (Figure 5B, panel b). AZU-1 up-regulation was not seen in 2-dimensional T4-2 monolayers treated with either of the functional inhibitors (data not shown). These findings suggest that AZU-1 expression is coupled to b1 integrin and EGFR signalling pathways in HMT-3522 cells cultured in a 3-dimensional context.

DISCUSSION

Using the genetically-paired HMT-3522 human breast progression series, we have identified a novel gene, AZU-1, that is expressed abundantly in non- and pre-malignant mammary epithelial cells (both primary and immortalized) but is dramatically down-regulated in a variety of breast carcinoma cell lines and carcinomas in situ. Restoration of AZU-1 expression to levels comparable to those seen in non-malignant S1 cells is sufficient to reduce the tumorigenic phenotype of the T4-2 cells, both *in vivo* and in culture. Our data suggest that re-expression of AZU-1 not only reduces the growth potential of these tumor cells but also restores their ability to form normal tissue structures in the context of 3D rBM assays. The expression patterns and functional properties of AZU-1 are consistent with the interpretation that AZU-1 is a candidate breast tumor suppressor gene that may play a role in the final events of tumorigenic conversion.

AZU-1 as a tumor suppressor

Two classes of tumor-suppressor genes are emerging from the study of cancers of the breast and other tissues. According to the broad terminology used by Sager and colleagues (Sager, 1997; Zhang et al., 1998), Class I genes include those mutated or deleted in cancers, such as BRCA-1 and BRCA-2 in the breast. Class II tumor suppressors, while displaying wild-type sequences, exert their phenotypic effects through dramatic changes in their expression profiles. Our current data on AZU-1 expression suggest that AZU-1 may be a tumor suppressor of the Class II type, although we have not yet analyzed breast tumors for possible mutations. However, in the HMT-3522 culture

model, the 4.4 kb AZU-1 transcript is effectively re-expressed in phenotypically-reverted T4-2 cells. Because of this, it is unlikely that these tumor cells incurred any gross genetic mutations or deletions that would destabilize or truncate the endogenous AZU-1 message. Because AZU-1 expression is elevated in both non-and pre-malignant cells, it is possible that the loss of AZU-1 expression is a decisive and causal step in the process of tumorigenic progression.

As a putative tumor suppressor, AZU-1 joins the ranks of a collection of gene products with diverse cellular functions. In addition to genes that function to regulate proliferation and gene transcription, examples of Class II suppressors include proteins that determine the overall morphological and adhesive properties of cells: proteins involved in cell-matrix interactions (a6 integrin) (Sager et al., 1993), cell-cell communication (E-Cadherin, connexins 26 and 43) (Alford and Taylor-Papadimitriou, 1996; Hirschi et al., 1996) and intracellular cytoskeleton (cytokeratins, gelsolin) (Sager, 1997; Mielnicki et al., 1999). Another previously identified tumor suppressor of this class is maspin, a serine protease inhibitor (serpin), that, like AZU-1, is expressed in both luminal epithelial and myoepithelial cells (Zou et al., 1994; Sager et al., 1997). While the functional significance of AZU-1 expression in myoepithelia is not clear at this time, it is possible that AZU-1 performs common functions in both epithelial cell types and that misregulation of AZU-1 expression in myoepithelial cells may also contribute to tumor progression (Ronnov-Jessen et al., 1996; Pechoux et al., 1999).

AZU-1 related genes

AZU-1 shares overall sequence similarity with three genes reported in GenBank, called TACC1 (Still et al., 1999), TACC2 and TACC3 (Still et al., 1999). Comparison of AZU-1 and TACC2 sequences reveals that these two gene products, with the exception of three distinctions (two insertions, 4 and 47 amino acids long, and one amino acid substitution), are identical. Moreover, the AZU-1 gene maps to chromosome 10q26 (Kuo and Gray, unpublished results), a site analogous to the one reported for the TACC2 gene (Still et al., 1999). Whether the differences between AZU-1 and TACC2 sequences are due to differential splicing or to variations in cloning procedures is not clear. However, it is unlikely that the additional sequences found in TACC2 are required for AZU-1's tumor suppressor function since the cDNAs used in our studies were sufficient to reduce the tumorigenic phenotype. Based on our results showing a tumor suppressive, rather than a cell transforming, effect on cells, we propose that the name AZU-1 be adopted as the preferred nomenclature for this gene.

TACC1, cloned from the 8p11 breast cancer amplicon, exhibits considerable homology with AZU-1 with respect to both its linear sequence and domain organization (Still et al., 1999). Similarities are most evident in AZU-1's SPAZ domain, Region I and the coiled-coil domain; Region II of AZU-1 is altogether absent in TACC1. TACC1 contains a 342 amino acid N-terminal extension that harbors at least one additional SPAZ domain. TACC3 (Still et al., 1999), while more similar to TACC1 than AZU-1, also contains a SPAZ domain and a C-terminal coiled-coil domain.

A potential role for AZU-1 in protein-protein interactions

Of the four predicted protein domains of AZU-1, two show structural conservation with previously characterized protein-binding motifs. One such domain is the SPAZ domain, located at the AZU-1's N-terminus. Because proline-rich regions have been shown to increase the apparent molecular mass of many proteins, including zyxin (Sadler et al., 1992) and kruppel (Ollo and

Maniatis, 1987), the aberrant migration of AZU-1 on SDS gels is likely attributable to its proline-rich SPAZ domain. Two invariant serines, found in all four SPAZ domains identified to date, may be important kinase recognition sites and thus targets for regulation through phosphorylation. Fold recognition studies, using the GenTHREADER program (Jones, 1999), indicate that AZU-1's N-terminal SPAZ domain is likely to possess an immunoglobulin (Ig)-like b-sandwich fold. For each SPAZ domain identified here, structural similarity with at least one known Ig-like structure was reported; the most reliable prediction was for the BCK1 SPAZ domain which matched an Ig-like domain in human Cd2 (estimated probability of correct match = .59). Ig-like domains are protein-binding motifs that are commonly found in the extracellular ligand-binding portions of cytokine and cell-adhesion receptors (e.g., FGFR and TCR) (Givol and Yayon, 1992; Smith and Xue, 1997). They are also prominent protein-binding motifs in a number of intracellular proteins including the structural muscle protein, titin (Improta et al., 1998). Based on these sequence predictions, the SPAZ domain is possibly a new member of the immunoglobulin superfamily and as such may function as a protein-binding interface.

A coiled-coil domain (CCD) is predicted at the C-terminus of AZU-1. CCDs form amphipathic helices that associate with other CCDs to form superhelical bundles of 2-5 protein subunits (Lupas, 1996; Lupas, 1997). Originally observed in large structural proteins like myosin, fibrinogen and cytokeratins (Lupas, 1996), CCDs are now appreciated in a variety of molecular contexts including transcription factors like jun and fos (Glover and Harrison, 1995) and signal regulators such as APC (Joslyn et al., 1993). CCDs have been found in breast tumor suppressors as well. For example, Tsg101 contains a central CCD that is thought to mediate an interaction with the oncoprotein, stathmin, and influence microtubule dynamics during mitosis (Li and Cohen, 1996). While most closely related to TACC1 and TACC3, the CCD of AZU-1 shares notable similarity with the human gene, SB1.8 (DXS423E), a human homologue of the SMC1 protein of *S. cerevisiae* (Rocques et al., 1995). SMC1 belongs to a family of myosin-like genes, called cohesins, that regulates chromosome segregation during mitosis; mutations in SMC1 give rise to chromosomal non-disjunction or total chromosome loss, both of which could contribute to genome instability and perhaps tumor progression (Michaelis et al., 1997). Whether the CCD of AZU-1 performs similar functions remains to be determined.

Our predictions indicate that the coiled-coil region of AZU-1 is best-suited for the formation of dimers. Conceivably, this region may support the formation of AZU-1 homodimers, or possibly heterodimers with similarly proportioned coiled-coil domains, such as those found in the TACC1, TACC3 or SB1.8. Given that overexpression of TACC1 results in cell transformation (Still et al., 1999), while high AZU-1 expression suppresses tumor growth, it is interesting to speculate that interactions between these two molecules may be required for properly regulated cell growth and tissue morphogenesis. Subtle changes in TACC1:AZU-1 ratios may potentiate the tumorigenic process. Regardless of its specific partner, AZU-1, by virtue of both its SPAZ and coiled-coil domains, may enable protein-protein interactions that mediate localization, complex assembly and/or regulation of signalling events.

Coupling AZU-1 expression with b1 integrin and EGFR activities

Recently, we demonstrated that inhibition of either b1 integrin or EGFR function was sufficient to promote phenotypic reversion of T4-2 cells in 3D rBM assays (Wang et al., 1998). Regardless of the inhibitory agent used, phenotypic reversion was accompanied by the down-

regulation of both b1 integrin and EGFR proteins to levels observed in non-malignant cells. Evidence presented here suggests that AZU-1 gene expression is also coordinately regulated by b1 integrin and EGFR function at the level of its transcription. In T4-2 cells reverted with b1 integrin or EGFR inhibitors, AZU-1 expression is restored to levels comparable to those observed in non-malignant cells. The fact that AZU-1 up-regulation was not induced in T4-2 cells grown as monolayers suggests that the coordinate modulation is dependent upon the formation of tissue-like structures in the 3D rBM assays. Given that overexpression of AZU-1 is also sufficient to cause phenotypic reversion of T4-2 cells, it is possible that AZU-1 engages in an integrated cross-talk with the cell surface receptors, b1 integrin and EGFR. Thus, the tumorigenic conversion of the HMT-3522 cells would require the collective disruption of all of these coordinately-regulated elements. As such, AZU-1 may provide essential clues for understanding how cellular structure and the differentiated breast phenotype may be coupled.

Collectively, our data are consistent with a role for AZU-1 as a structural tumor suppressor gene that is misregulated during the final stages of breast tumor progression. Sequence predictions indicate that AZU-1 harbors a number of specific protein-binding interfaces, two of which have been found in proteins serving structural functions. Thus, it is possible that in normal cells, AZU-1-mediated protein-protein interactions contribute to the integrity of epithelial form and function. A reduction in AZU-1 levels may destabilize cellular architecture, thereby facilitating malignant conversion.

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Table 1. *In vivo* tumorigenicity of HMT-3522 cell lines.

Cell Type	Number of injection sites[1]	Number of Sites with Tumors[2]	Mean Tumor Size (mm^3) ± S.E. (n)
S1	32	0	0 (0)
T4-2	32	28	250 ± 80 (28)
T4-2 + vector	32	28	265 ± 95 (28)
T4-2 + AZU-1	32	4	38 ± 19 (4)

[1] Two injection sites per mouse; 16 mice in each group
[2] Lump > 10 mm^3

FIGURE LEGENDS

Figure 1. The AZU-1 gene is differentially expressed in non-malignant and tumorigenic human breast cells. Northern blot analysis was performed on total RNA (20 mg/lane) from breast cell and tissue extracts using ^{32}P-labelled AZU-1-specific probes. **A**, Comparison of AZU-1 expression in S2 and T4-2 cells detected with the 180 bp differential display cDNA probe. Two lower-abundance transcripts are indicated by small arrows; the presence of these bands was not always reproducible. **B**, AZU-1 expression in normal primary luminal epithelial and myoepithelial cells and in non-malignant breast cell lines, HMT-3522-S1 and MCF10A. **C**, Compared to S2 cells (lane 1), AZU-1 expression is reduced in a number of breast carcinoma cell lines: 2, T4-2; 3, HMT-3909; 4, MCF-7; 5, CAMA-1; 6, BT-20; 7, MDA-MB-468; 8, SKBR-3; 9, T47D; 10, MDA-MB-231; 11, Hs578T; 12, BT549. * = HMT-3909 cells display partial myoepithelial differentiation (Petersen et al., unpublished result). **D**, AZU-1 expression in tissues derived from normal breast (lane 1) and three carcinomas *in situ* (lanes 2-4). For **B-D**, an AZU-1 coding region probe was used; in all cases, a GAPDH probe was used as a loading control.

Figure 2. The sequence and structure of AZU-1. **A**, Deduced amino acid sequence of the AZU-1 571 amino acid open reading frame. 4 structural domains, labeled SPAZ, Region I, Region II and CCD, are boxed and two predicted NLS motifs are underlined. The N-terminal peptide used to generate the AZU-1 antibody is highlighted in gray. The "HATDEEKLA" sequence, a peptide that conserved between AZU-1 and TACC1, appears in black. **B**, Domain organization of AZU-1 and two AZU-1 related genes, TACC1 and TACC3. Based on its similarity with TACC1 and TACC3, AZU-1 can be partitioned into 4 domains: 1) the N-terminal serine and proline-rich AZU-1 (or SPAZ) domain, 2) Region I, a region that shares a moderate sequence similarity with TACC1 and to a lesser extent with TACC3, 3) Region II which is totally absent in TACC1 and partially removed from TACC3, and 4) the C-terminal coiled-coil domain. **C**, Sequence alignments of SPAZ domains from AZU-1, TACC1 (2 copies, a and b), TACC3 and BCK1 from *S. cerevisiae*. Residues that are conserved in three or more of these sequences appear in black; the corresponding columns are marked with an open circle. Two invariant serine residues are indicated by filled circles. Fold recognition analyses predict that SPAZ domains adopt immunoglobulin-like folds. **D**, Coiled-coil domain (CCD) sequence alignments of AZU-1, TACC1, TACC3 and SB1.8/DXS423E. Amino acid identities observed in two or more of the aligned sequences are indicated in black; in cases where two pairs of identical amino acids are observed in the alignment, AZU-1-like sequences are preferentially highlighted. The CCD heptad repeat positions, a-g, are indicated in brackets above the three regions where all four proteins fall into register. Positions a and d, often occupied by hydrophobic residues, are indicated in capital letters. Sequence identities among all four proteins in this region are most notable in the second half of the CCD.

Figure 3. The AZU-1 protein migrates with an apparent molecular mass of 80kDa on SDS-polyacrylamide gels. **A**, *In vitro* transcription/translation reactions were performed with ^{35}S-methionine in the absence (lane 1) or presence of luciferase cDNA (lane 2, positive control at 61 kDa) or AZU-1 cDNA (lanes 3-5). In lanes 1-3, 5 ml of whole lysate were loaded in each lane. The remaining AZU-1 lysate was immunoprecipitated with either pre-immune (lane 4) or AZU-1-

specific (lane 5) rabbit sera and the precipitated samples were loaded into adjacent wells. The resolved protein products were analyzed by autoradioagraphy. The AZU-1 cDNA gives rise to a single predominant protein with a M_r of 80 kDa. **B**, Protein extracts from S1 and T4-2 monolayer cultures (20 mg/lane) were analyzed by western immunoblotting using pre-immune (lanes 1 and 2) or anti-AZU-1 (lanes 1'and 2') rabbit sera. E-cadherin antibodies were used to control for protein loading. Like the *in vitro* translated protein, cellular AZU-1 migrates with an apparent molecular weight of 80 kDa by SDS-PAGE. On average, T4-2 cells exhibit a three-fold reduction in AZU-1 protein levels in comparison to non-malignant S1 cells.

Figure 4. Re-expression of AZU-1 in T4-2 cells reduces their tumorigenicity *in vitro*. Northern (**A**) and western (**B**) blot analyses were performed to monitor AZU-1 levels in S1, T4-2 control cells and AZU-1-infected T4-2 cells. AZU-1 expression is increased at both the RNA and protein levels upon introduction of the AZU-1 transgene into T4-2 cells (in both cases ~2-3-fold). A GAPDH probe and an E-cadherin antibody were used as loading controls in northern and western blots, respectively. *In vitro* tumorigenicity of the various HMT-3522 cells was measured in soft agar assays (**C**) and in invasion assays (**D**). In both cases, overexpression of AZU-1 in T4-2 cells gave rise to reduced tumorigenic behavior (i.e., reduced anchorage independent growth and reduced capacity to migrate through a basement membrane-like gel). The data presented here represent the averages of 3 independent experiments and correspond to the mean activity of triplicate measurements $\pm$ standard error.

Figure 5. Increased AZU-1 expression levels correlate with phenotypic reversion in 3D rBM assays. **A**, AZU-1 induces phenotypic reversion. S1, T4-2 (vector-infected) and T4-2-AZU-1 cells were embedded as single cells in 3D rBM assays. After 10 days in culture, the colonies were measured (expressed as colony diameter in mm $\pm$ standard error) and imaged using phase microscopy (**a**, **b** and **e**). Cultures were immunostained with antibodies specific for Collagen IV (**c** and **f**) or b4 integrin (**d** and **g**). **B**, AZU-1 is re-expressed upon EGFR- and b1 integrin-induced phenotypic reversion. **a**, S1 and T4-2 cells were cultured in 3D rBM assays in the absence or presence of functional inhibitors of b1integrin (T4-2b1) or EGFR (T4-2tyr; tyr = tyrphostin). Unlike control cells, inhibitor-treated T4-2 cells exhibit an S1-like, acinar phenotype in 3D cultures. **b**, Total RNA harvested from these cultures was analyzed in northern blots using an AZU-1-specific probe. GAPDH was used as a loading control. AZU-1 expression is restored to S1-like levels in T4-2 cells that have undergone phenotypic reversion in the 3D rBM assay. Scale bars = 50 mm.

Figure 1

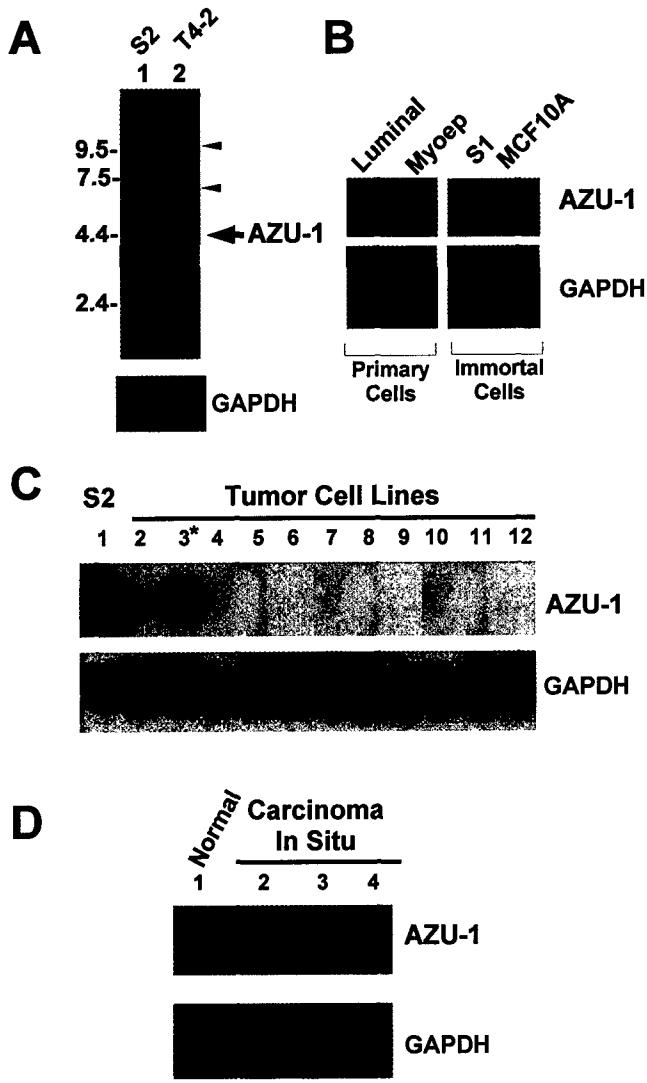
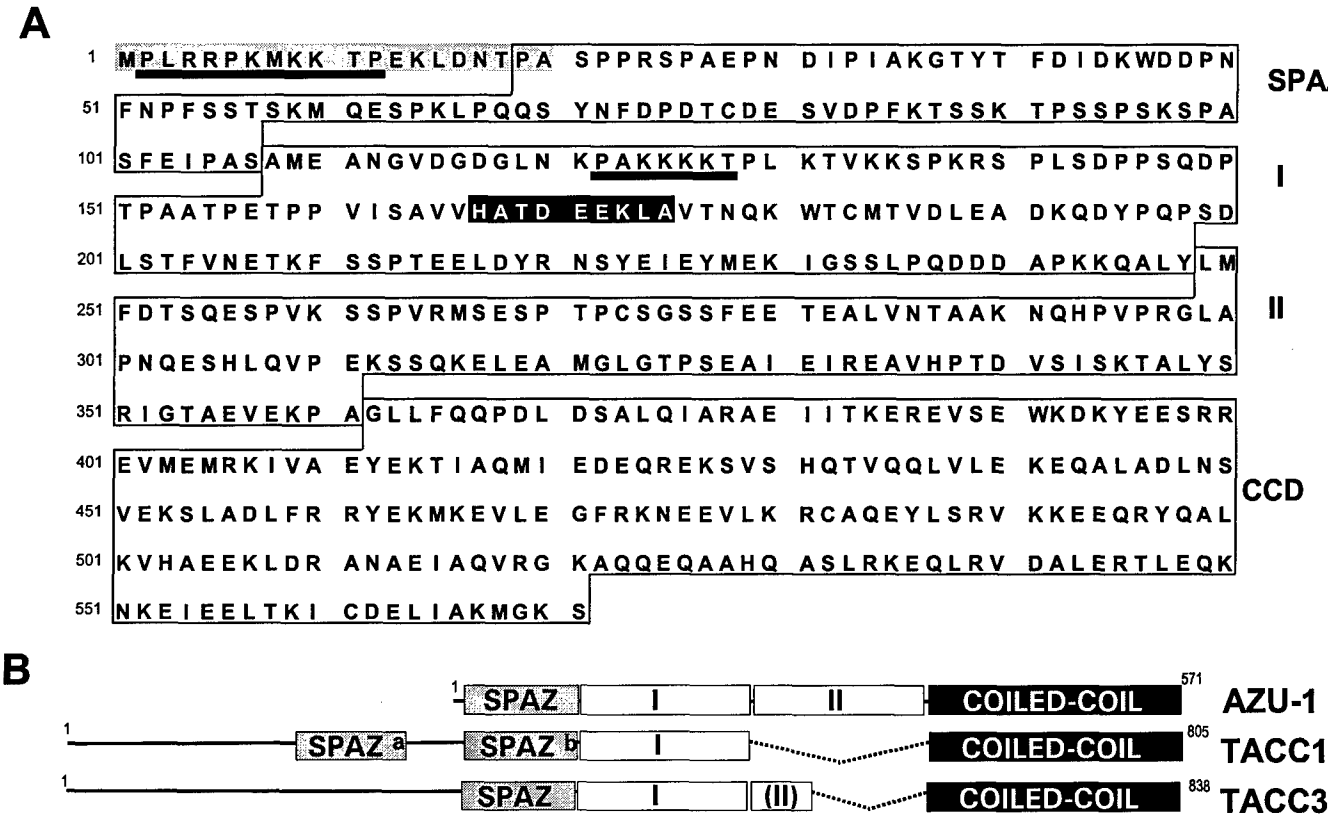
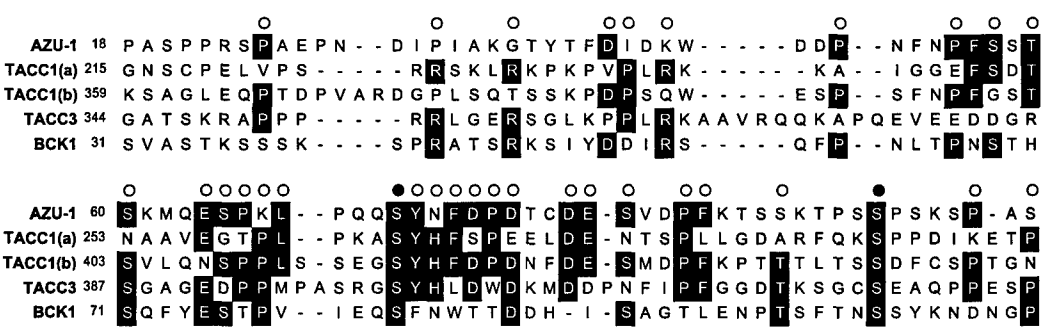

A
S2
T4-2
1
2
9.5-
7.5-
4.4-
2.4-
AZU-1
GAPDH
B
Luminal
Myoep
S1
MCF10A
AZU-1
GAPDH
Primary Cells
Immortal Cells
C
S2
Tumor Cell Lines
1 2 3* 4 5 6 7 8 9 10 11 12
AZU-1
GAPDH
D
Normal
Carcinoma In Situ
1 2 3 4
AZU-1
GAPDH

Figure 2



C SPAZ Domain



D Coiled-Coil Domain

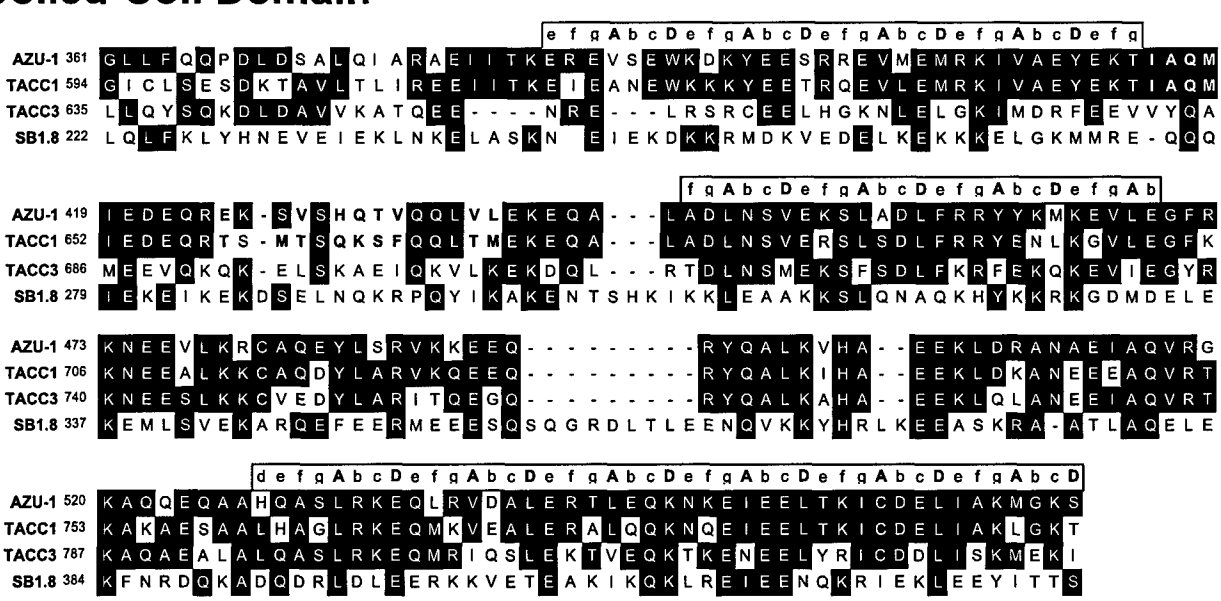
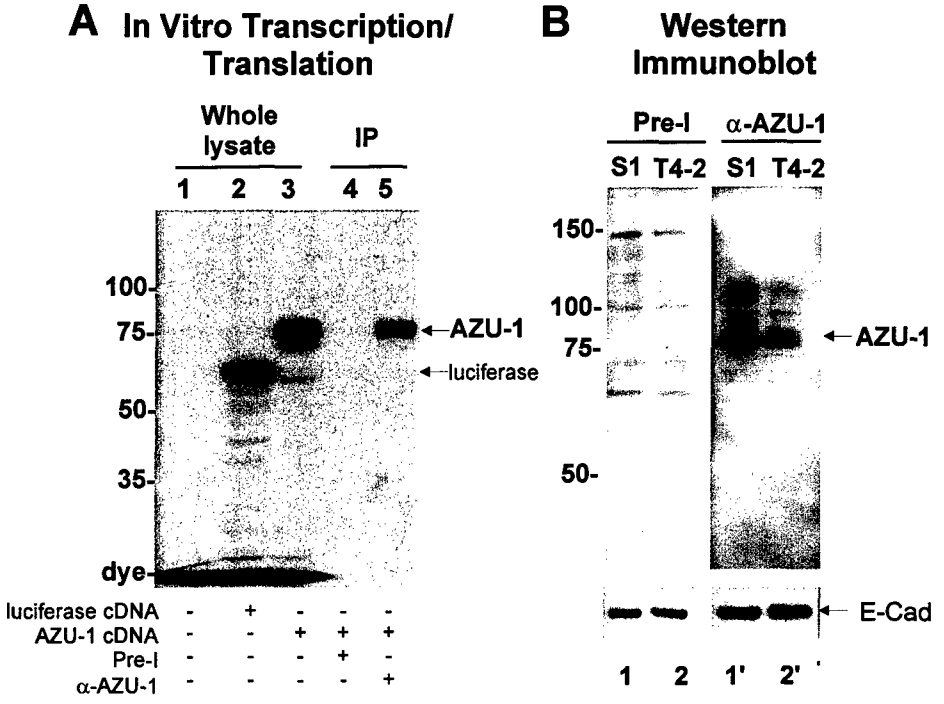


Figure 3


A
In Vitro Transcription/ Translation
Whole lysate
IP
1 2 3 4 5
100
75
50
35
dye
AZU-1
luciferase
luciferase cDNA - + - - -
AZU-1 cDNA - - + + +
Pre-I - - - + -
α-AZU-1 - - - - +
B
Western Immunoblot
Pre-I
α-AZU-1
S1 T4-2 S1 T4-2
150
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75
50
AZU-1
E-Cad
1 2 1' 2'

Figure 4

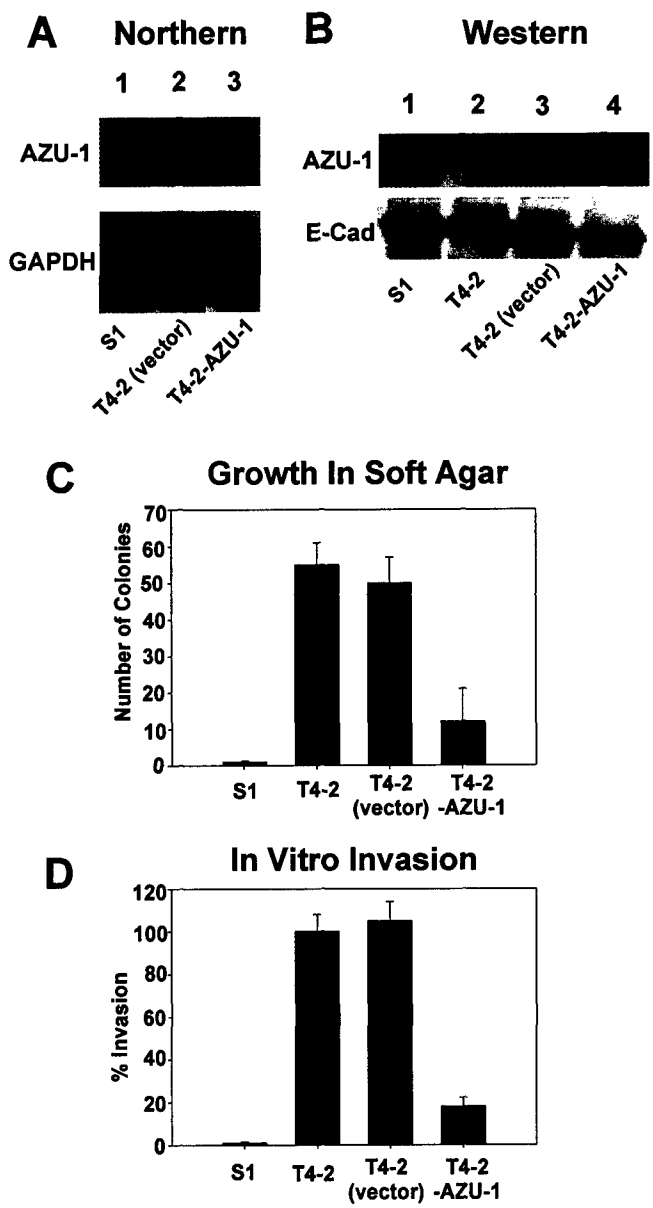
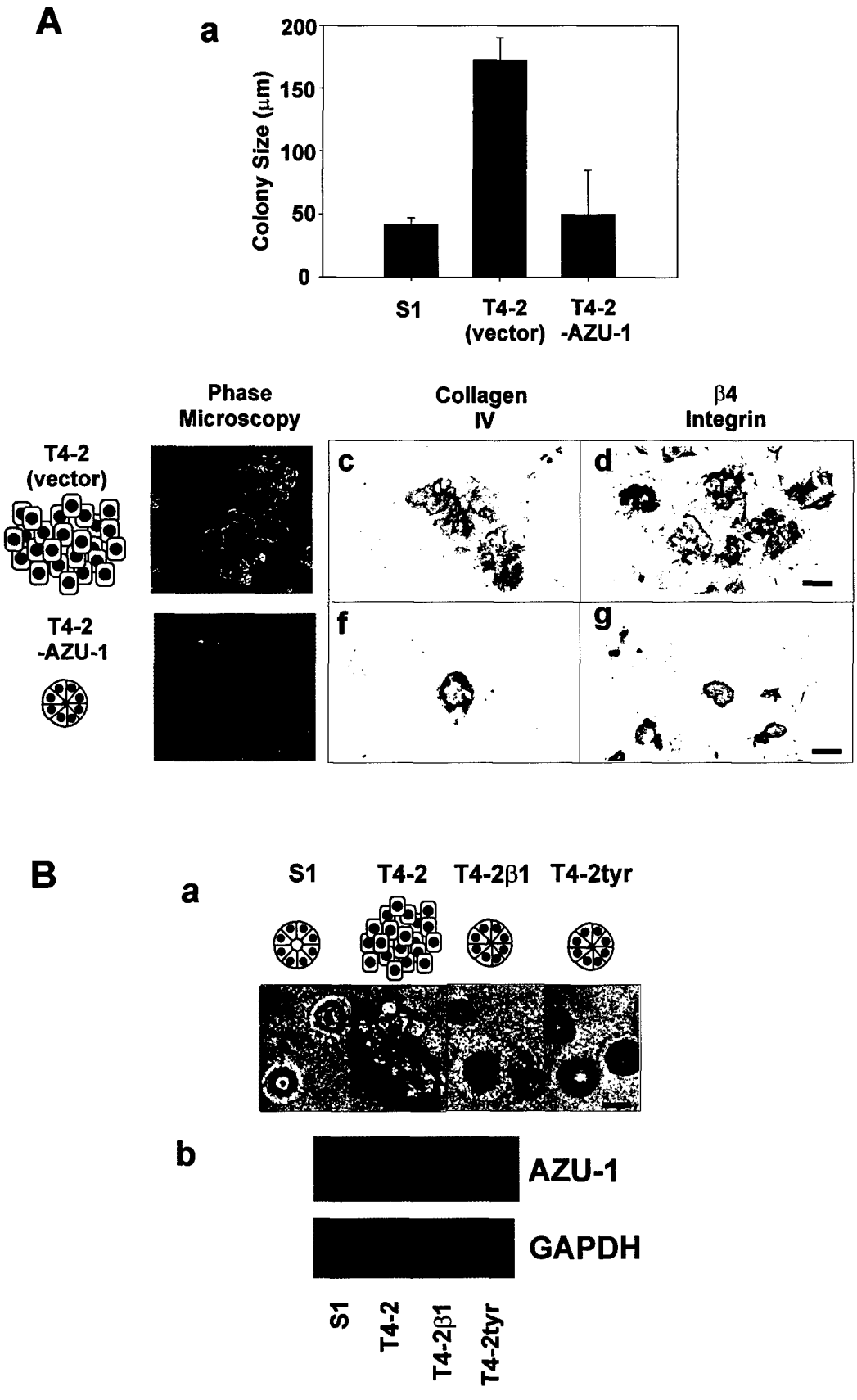

A
Northern
1 2 3
AZU-1
GAPDH
S1
T4-2 (vector)
T4-2-AZU-1
B
Western
1 2 3 4
AZU-1
E-Cad
S1
T4-2
T4-2 (vector)
T4-2-AZU-1
C
Growth In Soft Agar
Number of Colonies
70
60
50
40
30
20
10
0
S1
T4-2
T4-2 (vector)
T4-2 -AZU-1
D
In Vitro Invasion
% Invasion
120
100
80
60
40
20
0
S1
T4-2
T4-2 (vector)
T4-2 -AZU-1

Figure 5


A
a
Colony Size (μm)
200
150
100
50
0
S1
T4-2 (vector)
T4-2 -AZU-1
Phase Microscopy
Collagen IV
β4 Integrin
T4-2 (vector)
T4-2 -AZU-1
c
d
f
g
B
a
S1
T4-2
T4-2β1
T4-2tyr
b
AZU-1
GAPDH
S1
T4-2
T4-2β1
T4-2tyr